Single-cell microbe physiology assessed by flow cytometry

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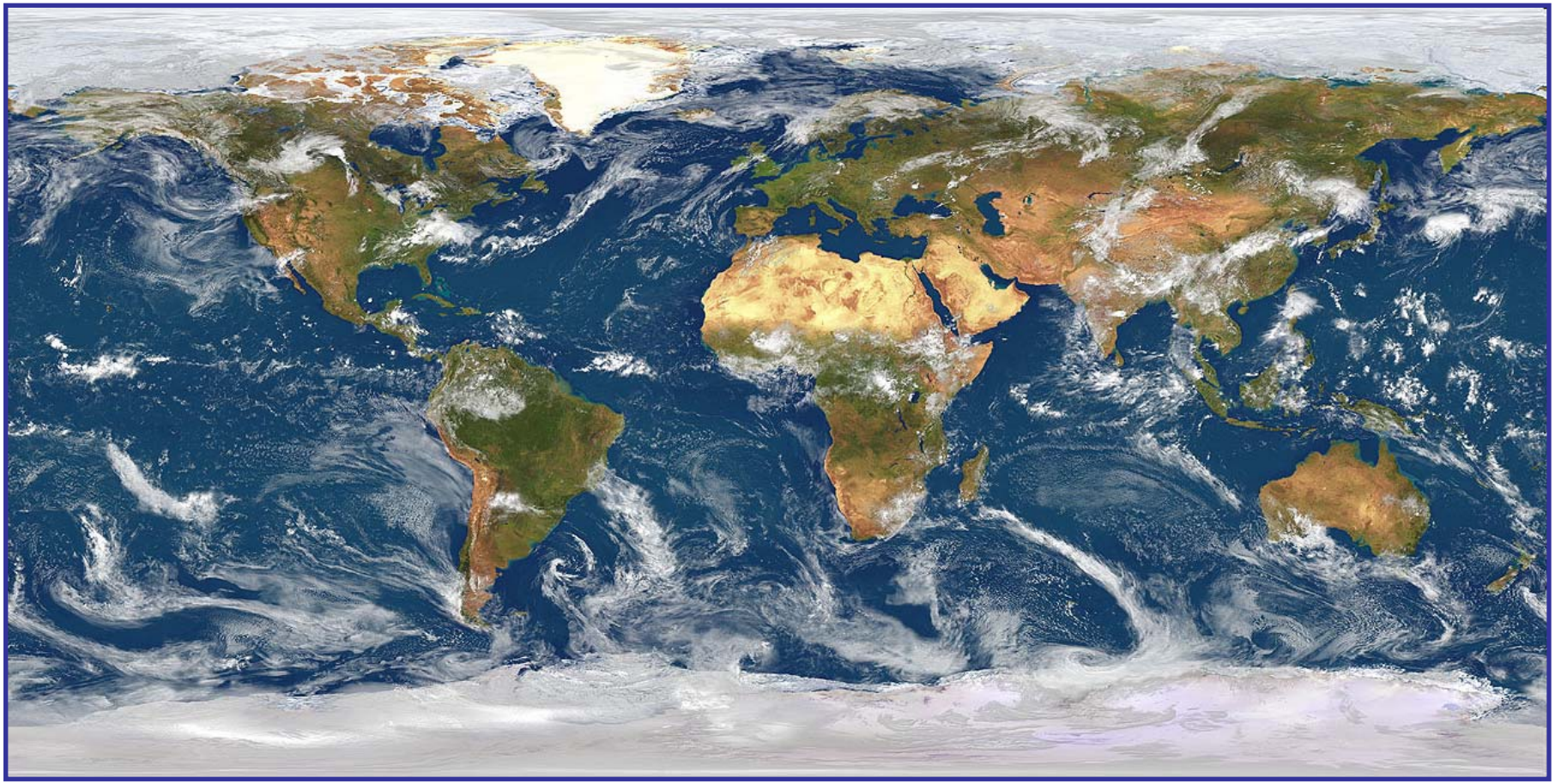
BD Biosciences Environmental Biology Workshop

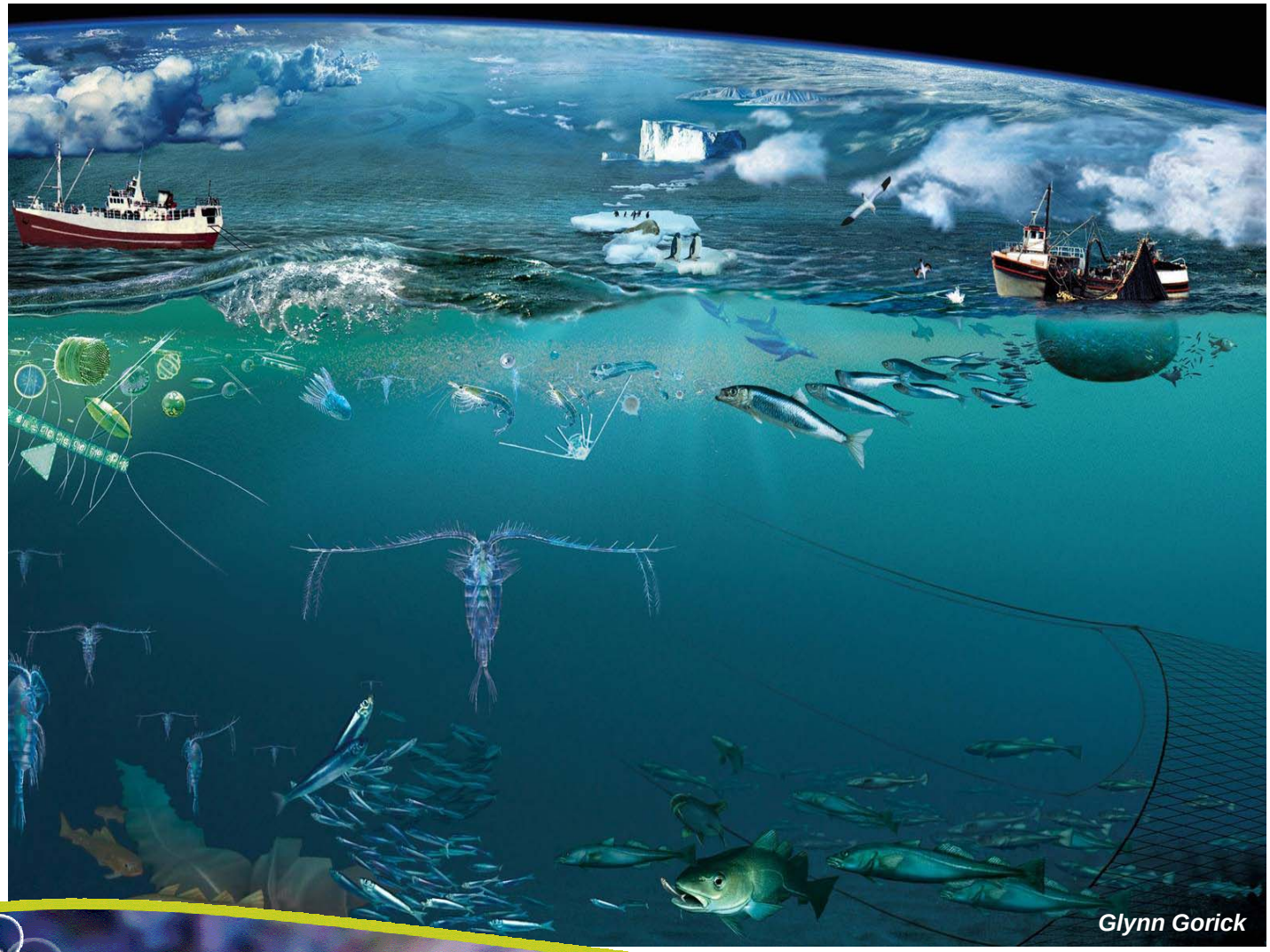
BD

Importance of the ocean



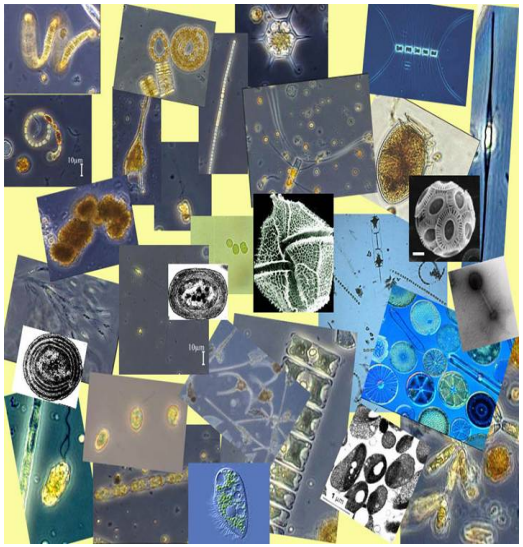
Hydrosphere ➔ >70% of the Earth

Prokaryotes:
the largest source of unknown biodiversity



Do you know?

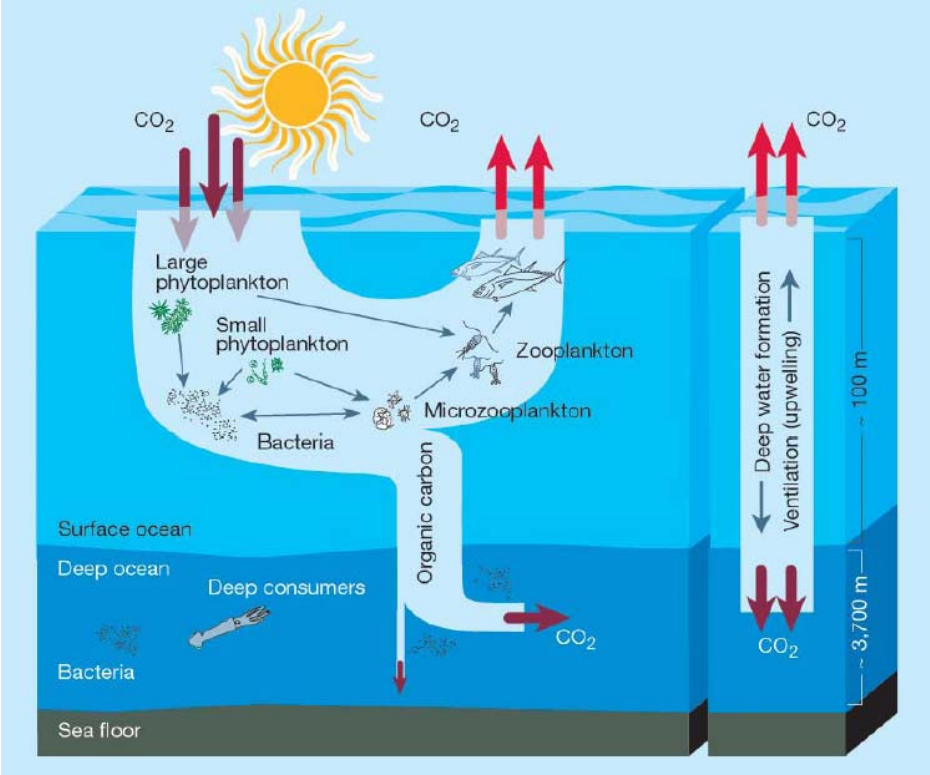
Aquatic unicellular microorganisms from 0.2 -100 µm
➔ 50% of the total biomass of the planet



	Pg Carbon (10^{15}g)
Phytoplankton (<20µm)	3 – 4
Bacteria (0.5-2 µm)	2.8 - 13.7
Virus (0.2 µm)	0.027 – 0.27
Whales	0.0041 – 0.012
Human beings	0.03

"Life on Earth is microscopic!" (*Sean Nee, 2004*)

Importance of aquatic microorganisms



Chisholm, 2000, Nature 407: 685-687

- Crucial roles in the functioning of the Earth's biosphere

- Dominate the marine ecosystem (biomass, high rate of turnover)

Responsible for :

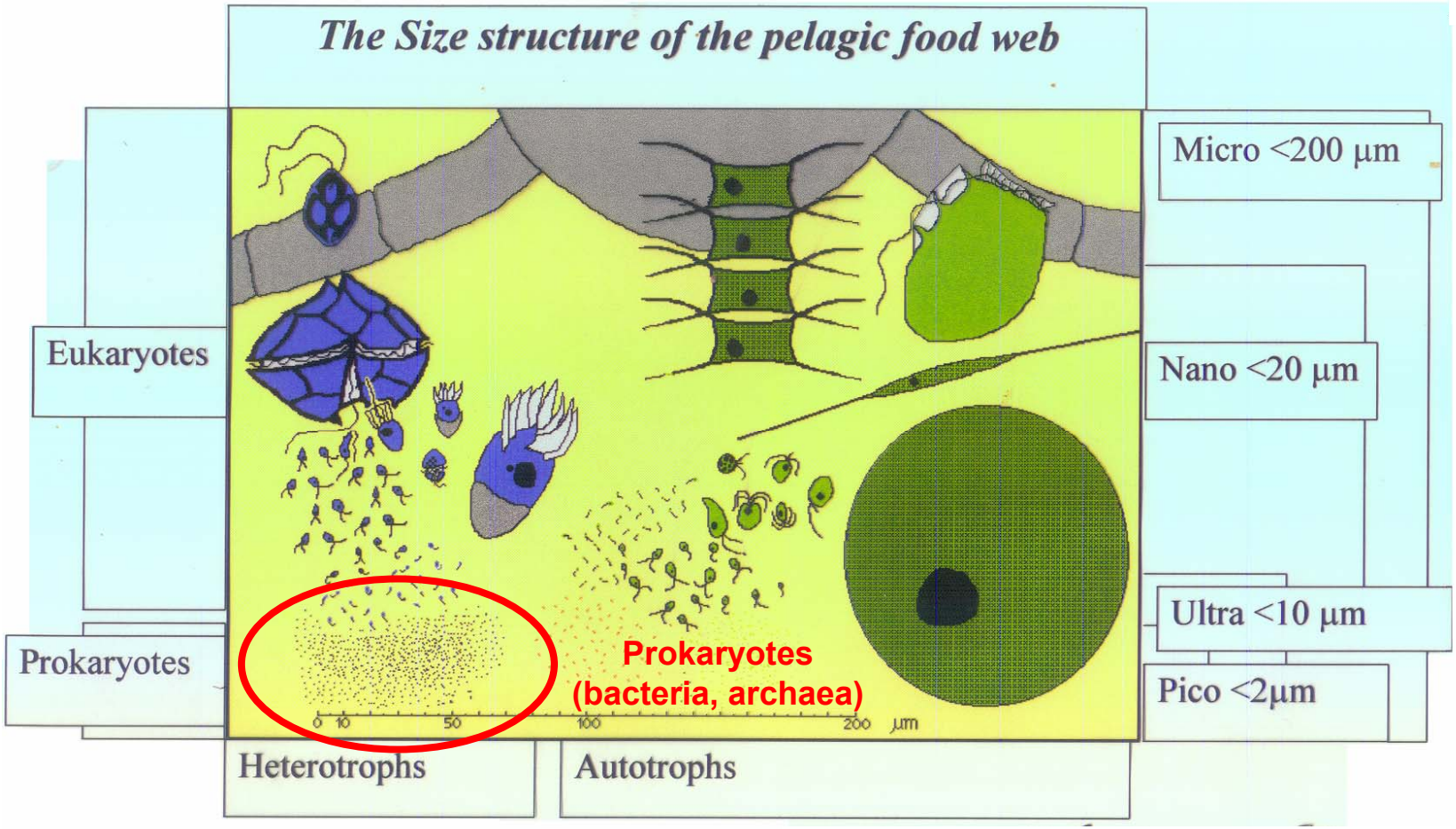
- (i) The production of organic matter (about half of our Planet's annual primary production) → CO_2 uptake

- (ii) Oceanic mineralization (water column) → CO_2 release

- (iii) Playing a role in regulating the climate (contribution to the atmospheric CO_2 sequestration in the deep ocean) ; producing chemically-active biogases

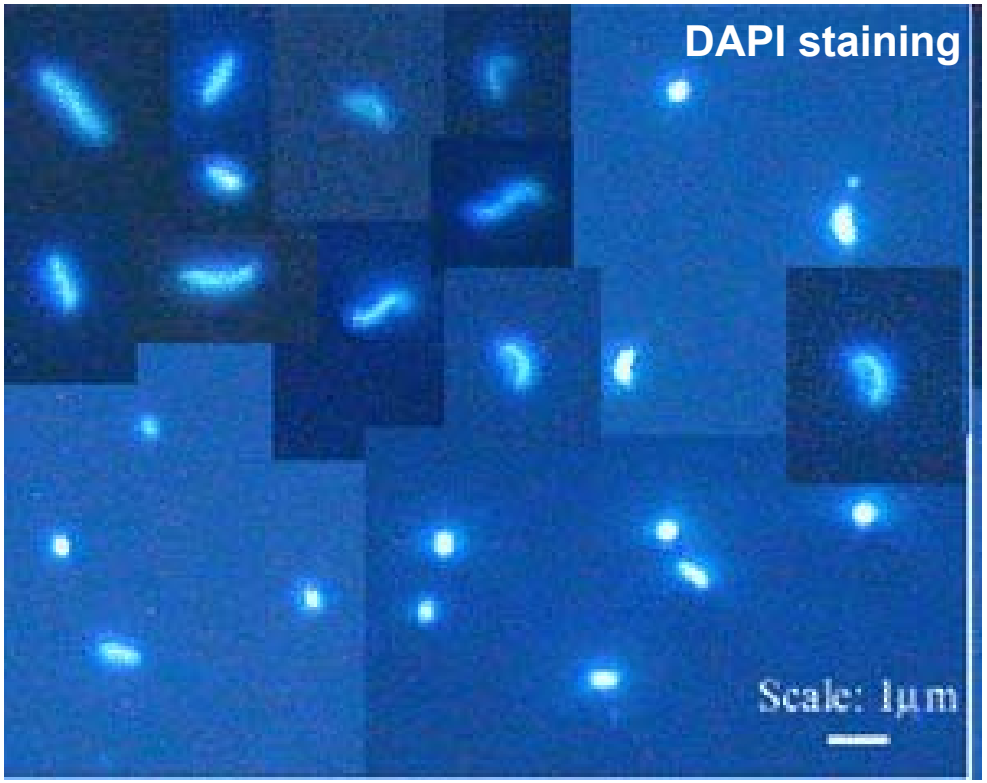
- (iv) Toxicity (ecosystem and sanitary risks)

Problems : Diversity and Size range



Dr. M. Reckermann FTZ

How do bacteria look like?



(F.J. Jochem)

Different morphotypes of pelagic bacteria after DAPI staining:

- **Cocci,**
- **Rod-shaped,**
- **Curved bacterie.**

How to characterize bacteria?

1. **Identification** (clusters, taxa, species?) ➔ biodiversity

2. **Abundances** and estimation of the **biomass**
   ➔ Spatio-temporal variability of populations (natural or induced)

3. **Physiological state** ➔ Heterogeneity
   ➔Viability (active, inactive, and dead cells)

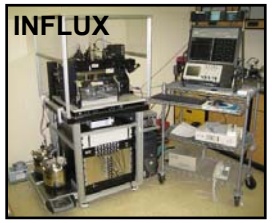
4. Qualify and quantify **metabolic** or **enzymatic** activities

➔ Single cell analysis

Flow Cytometry

Measure (-metry) of optical properties of cells (cyto-) transported by a liquid sheath (flow) to a light source excitation (most often a laser).

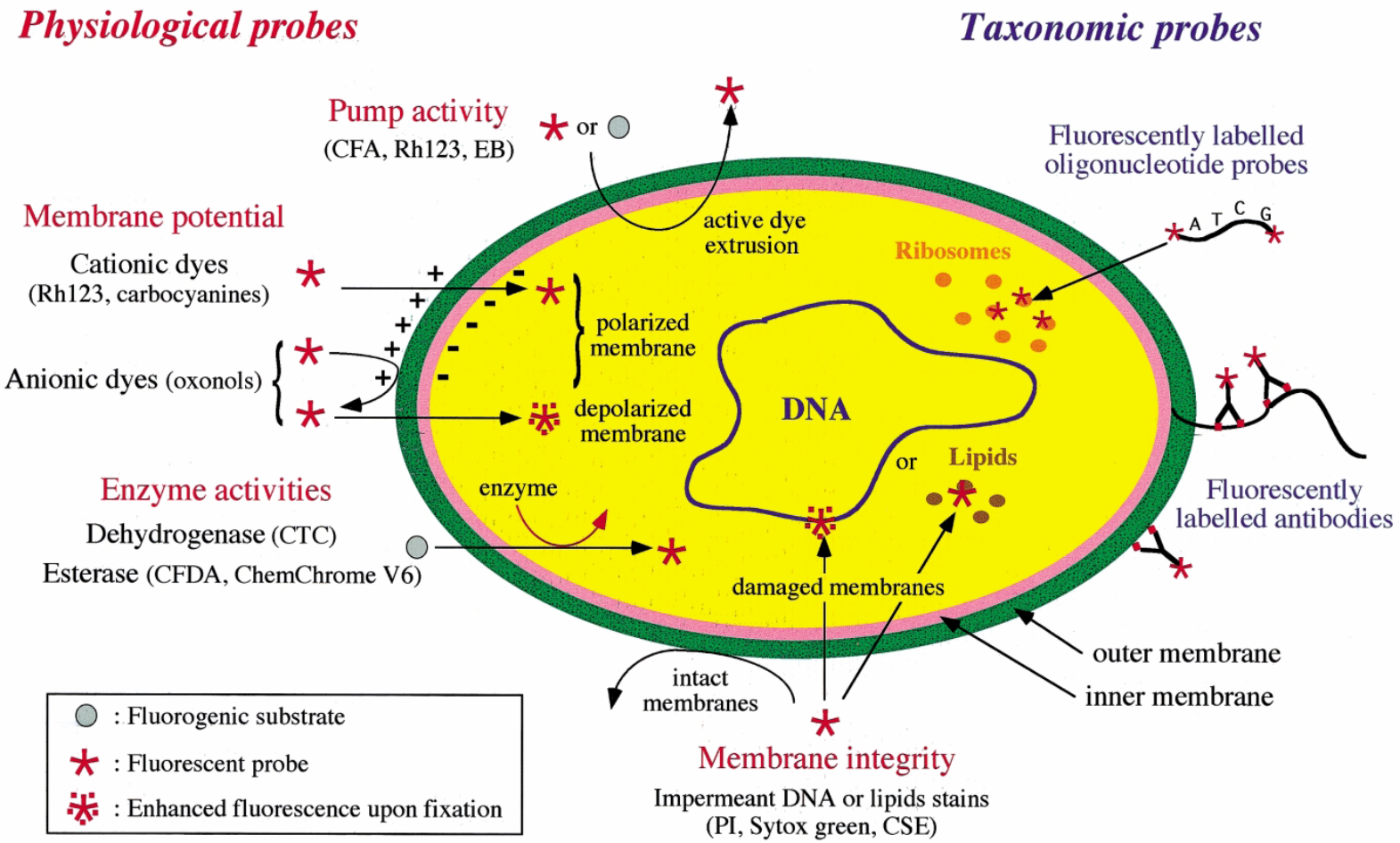
- **Light (laser or arc lamp) scattered by particles (cells)**
- **Natural or induced fluorescence(s) emitted by the particles (cells)**
- **Cells flow in single file**
- **Multivariate analysis**
- **Identification of sub-populations**



Why is flow cytometry so popular among microbiologists?

- **Fast analyses** (up to several thousands cells s^{-1})
  - ➔ Large amount of cells analyzed
  - ➔ Statistical results representative of the population
- **Multiparametric analyses at the single cell level**
  (several scatters and fluorescences)
- Quantitative data (correlated to biochemical data)
- **Real time** measurements
- **Size class distribution and cell abundance**
- **Unique identification markers** :
  - **natural** (chlorophyll, other pigments) → autofluorescence
  - **induced** (staining) → fluorochromes (dyes)
- **Cell sorting (post-analyses, cultures)**

Combining flow cytometry and fluorescent compounds

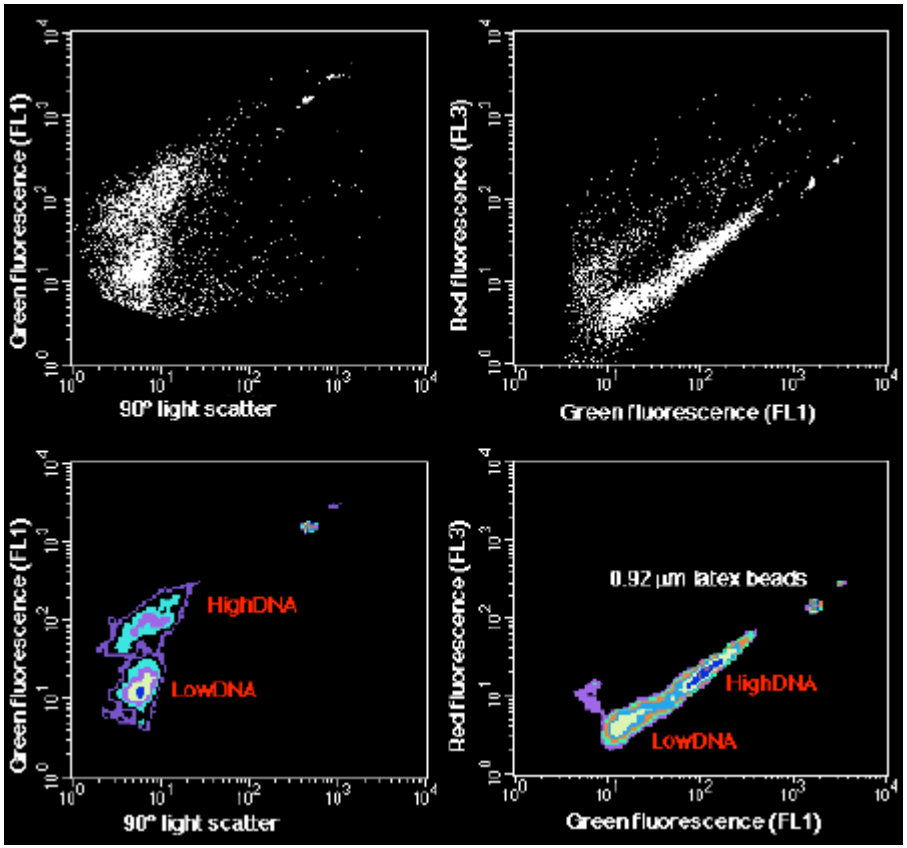


Joux *et al.*. Microbes and Infection, 2, 2000, 1523–1535

Bacteria analysis by flow cytometry : based on nucleic acid staining

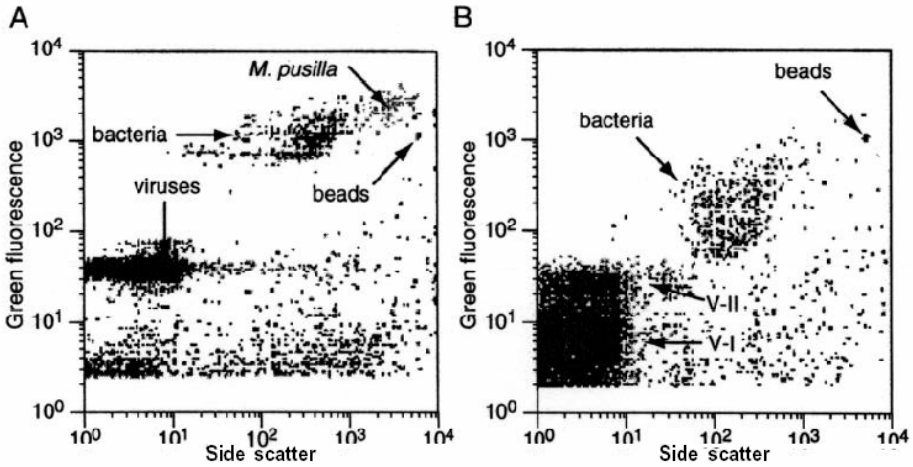
SYBR Green

Bacteria

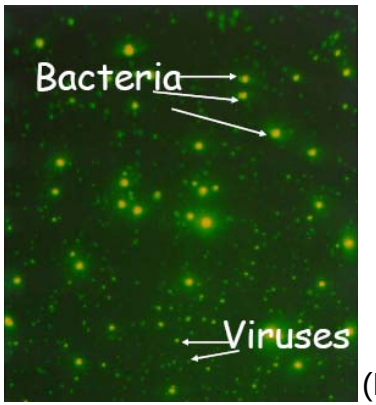


(J.Gasol)

Viruses

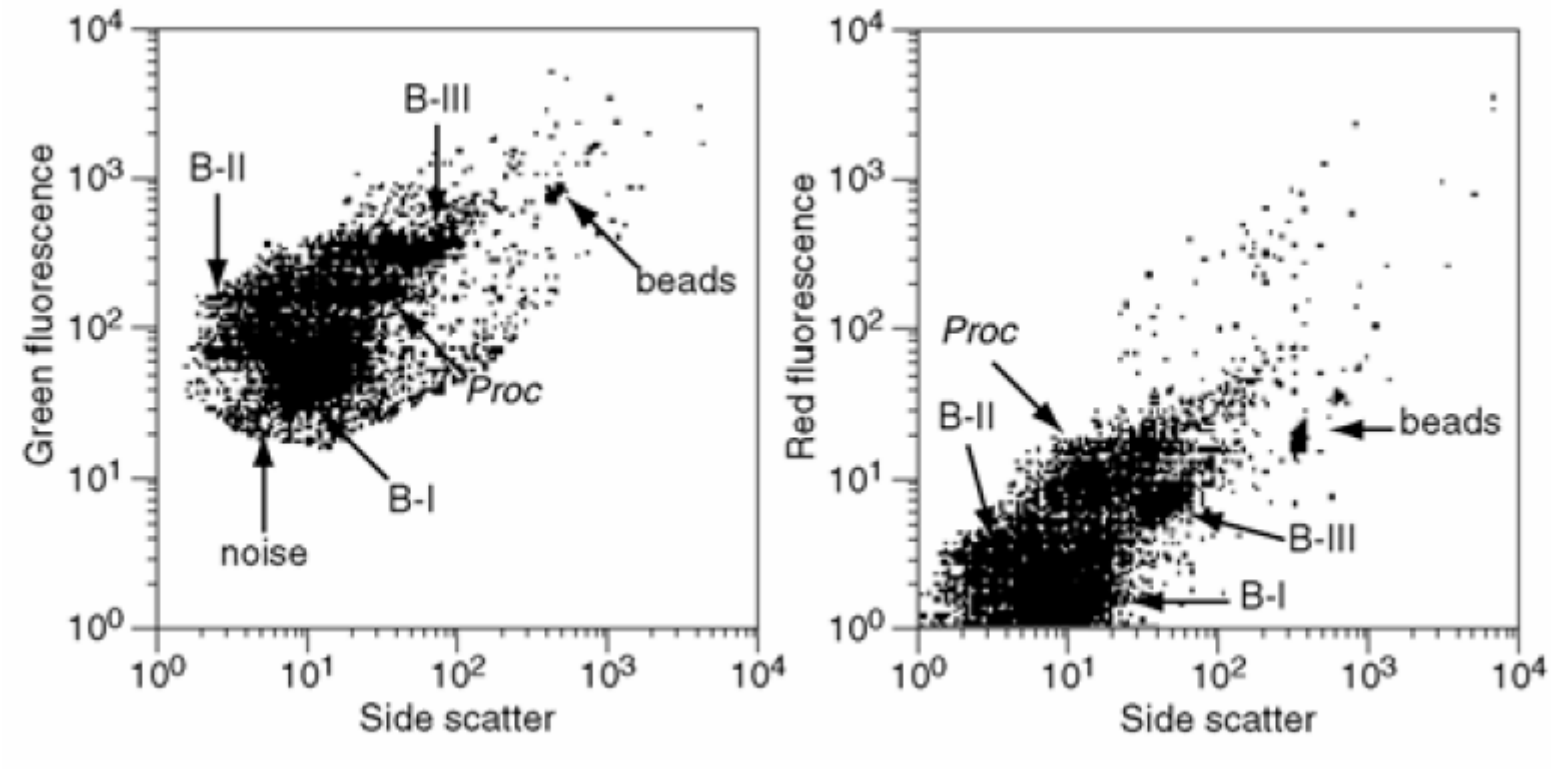


(D.Marie)



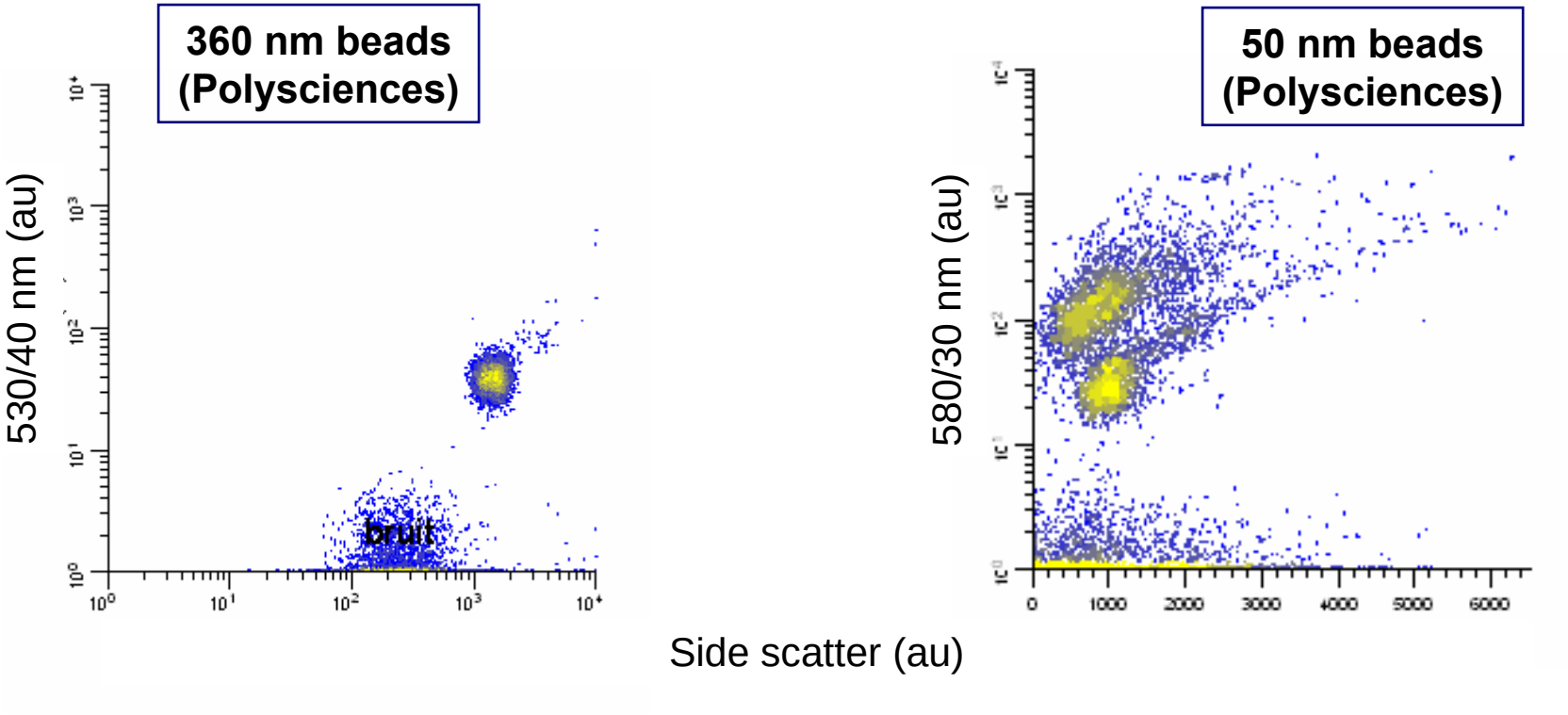
(M. Veldhuis)

Optical Resolution of « Flow Cytometry Clusters »



Enumeration of Phytoplankton, Bacteria, and Viruses in Marine Samples
Dominique Marie, Frédéric Partensky, Daniel Vaulot, Corina Brussaard
Current Protocols in Cytometry, Unit Number: UNIT 11.11

Better discrimination with new instruments?



- Better discrimination of smaller particles (cells)?
- Sorting?

Bacteria viability

Why is viability important to address?

- **Only viable cells are responsible for activities measured *in situ* by bulk methods**

- **Why cells are alive or not?**
  ➔ Factors (natural or anthropic)?

Nucleic Acid Double Staining protocol (NADS)

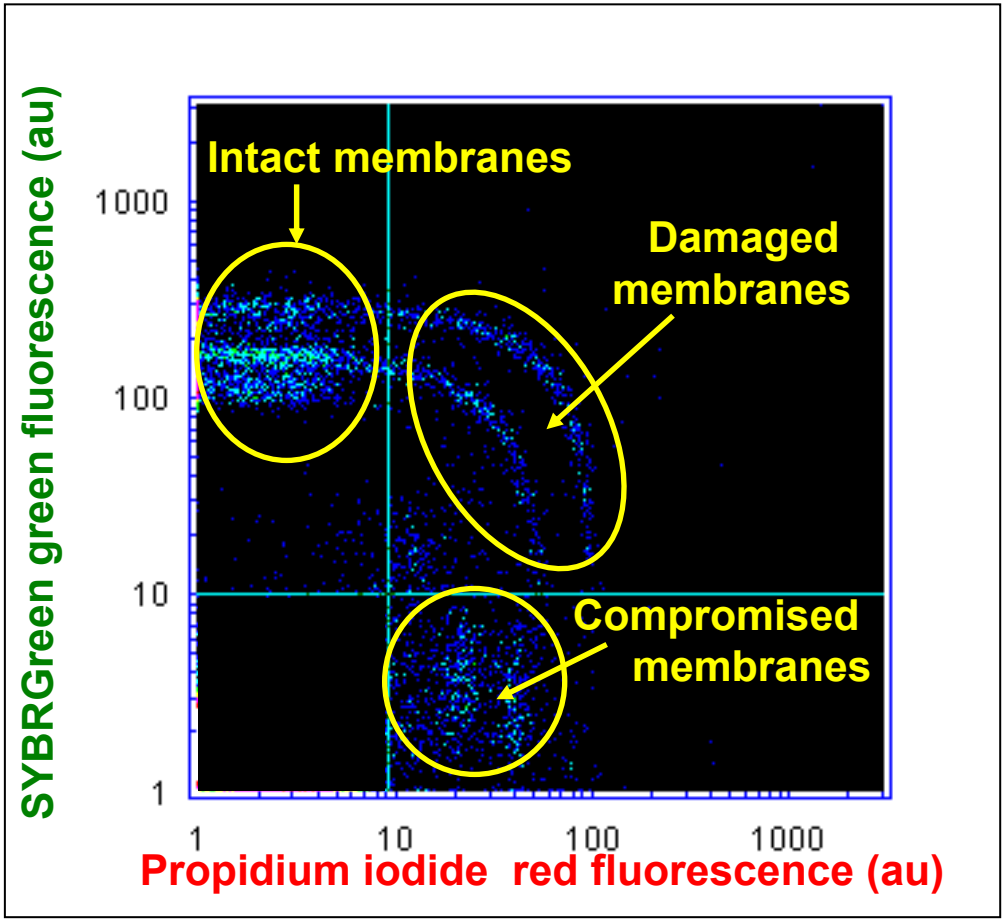
2 fluorochromes simultaneously :

- **SYBRGreen II** enters all membranes (intact and damaged cells)

- **Propidium iodide (PI)** enters only damaged and compromise membranes (dead cells)

Fluorescence resonance energy transfert (FRET) from SYBRGreen to PI

Viability assessed by FCM



Viable active/inactive cells

➔ **Cell cycle (cell division)**

➔ **Enzymatic activity**

Esterases, Phosphatases, Proteases, Peroxidases

➔ **Detection/quantification of a metabolic activity**

Ionic pumps (Ca)
Energy (transmembranar potential)
Respiration

Discrimination of bacteria based on the metabolic activity

Two types of methods to detect metabolically active cells:

Example of a method based on energy-dependent processes

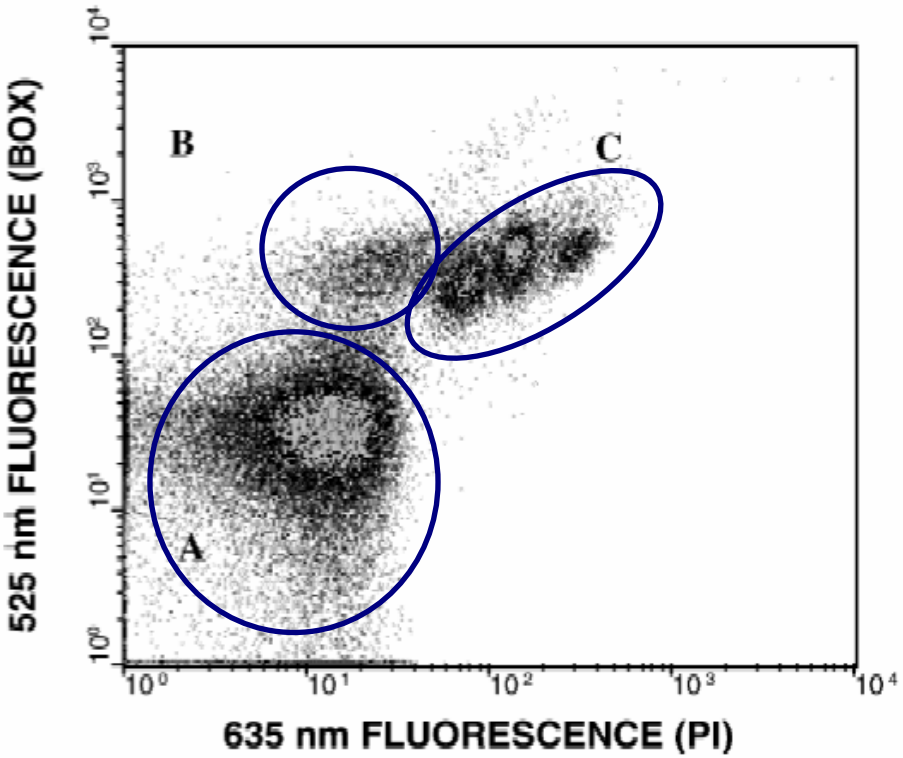
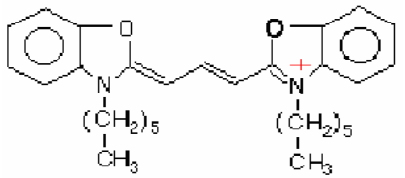


Fig. 9. Cell sample taken after 36 h during a high cell density fed-batch fermentation with *E. coli* W3110 stained with propidium iodide and bis-oxonol. Three main sub-populations of cells can be distinguished, corresponding to healthy cells (A), no staining, cells with no membrane potential (B), stained with *bis*-oxonol; and cells with permeablised membranes (C), stained with both propidium iodide and *bis*-oxonol (after Hewitt et al. (1999a); Hewitt et al. (1999b)).

From G . Nebe-von-Caron *et al.*, Journal of Microbiological Methods 42 (2000)

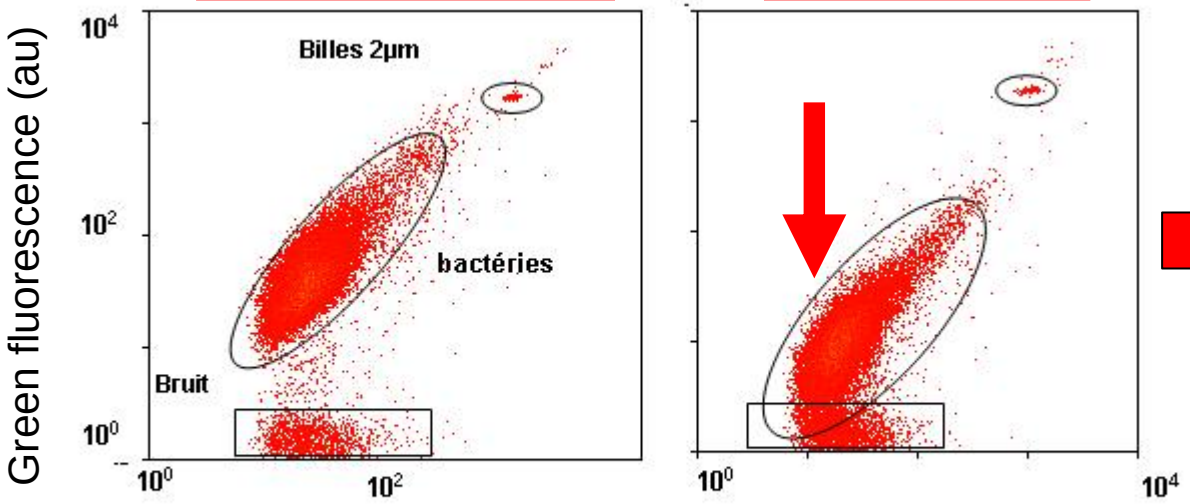
Example of a method based on energy-dependent processes

Membrane potential dye ➔ Carbocyanine ($DiOC_6(3)$)



Culture of *P. nautica*

No agitation **Agitation**



Measure of respiration rate

Oxygraph OROBOROS 2K

(Mebarek et al., in prep)

Which respiration do we measure?

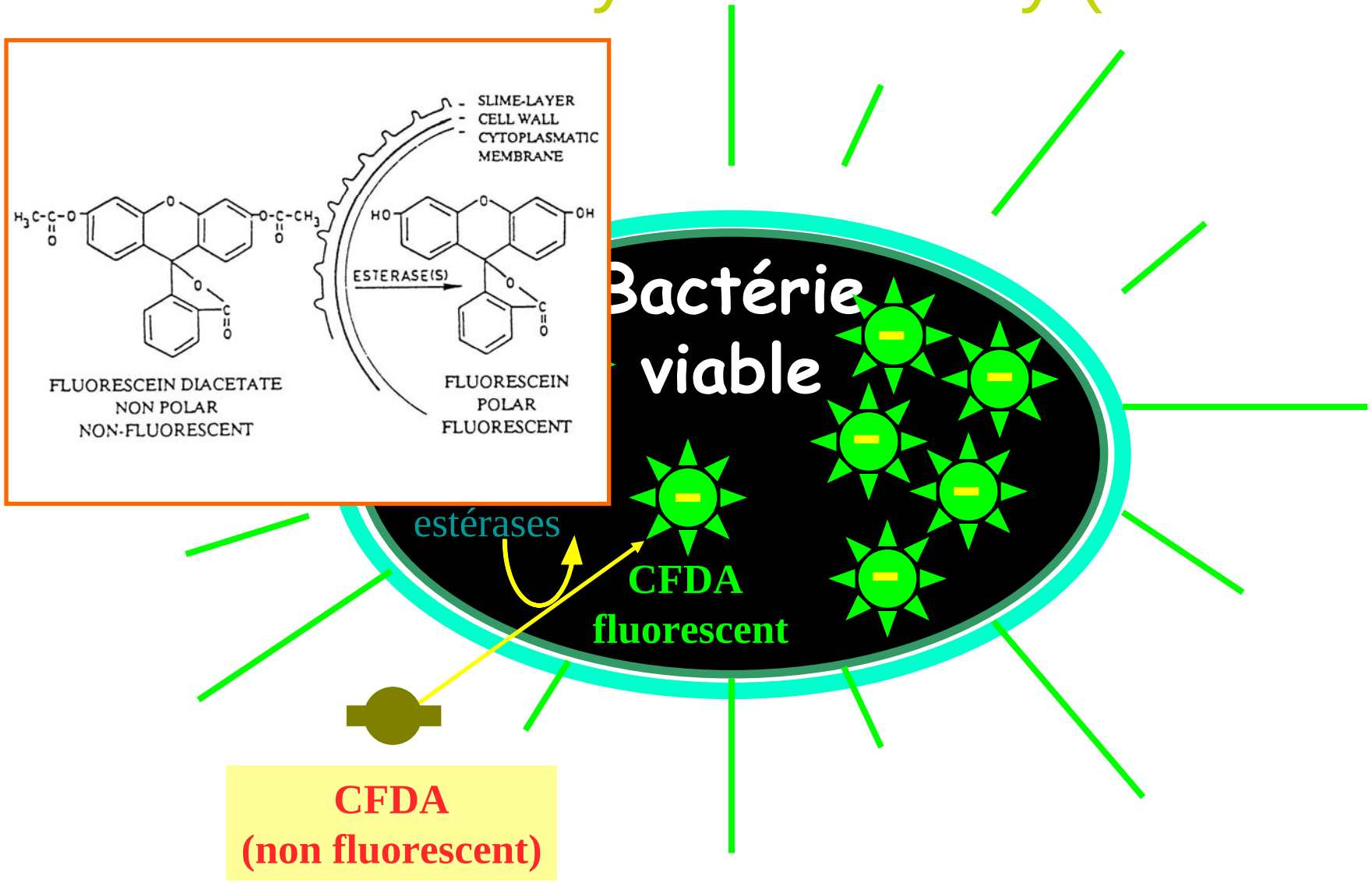
Example of method based on energy-independent processes

Enzymatic activity

- Esterase activity (FDA, CFDA, BCECF-AM, Calcein-AM, Chemchrome B)

- Dehydrogenase activity (CTC)

Detection of enzymatic activity (esterases)



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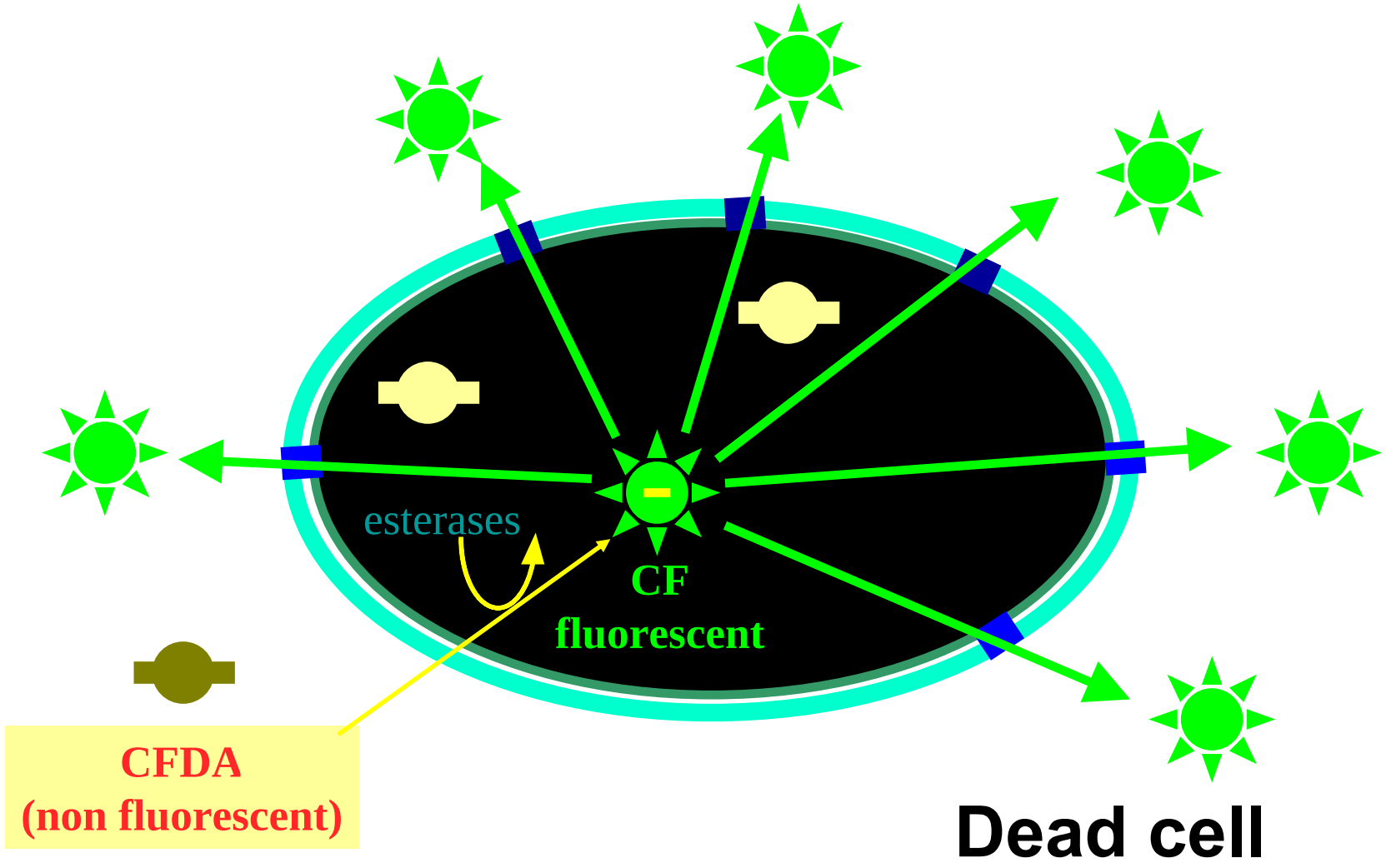

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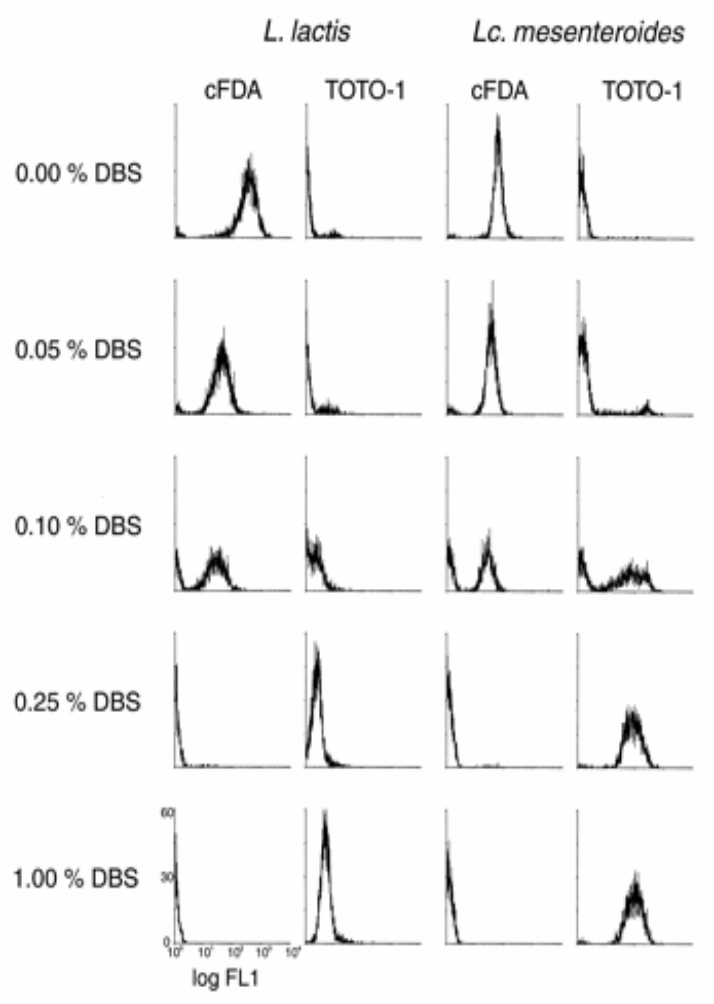
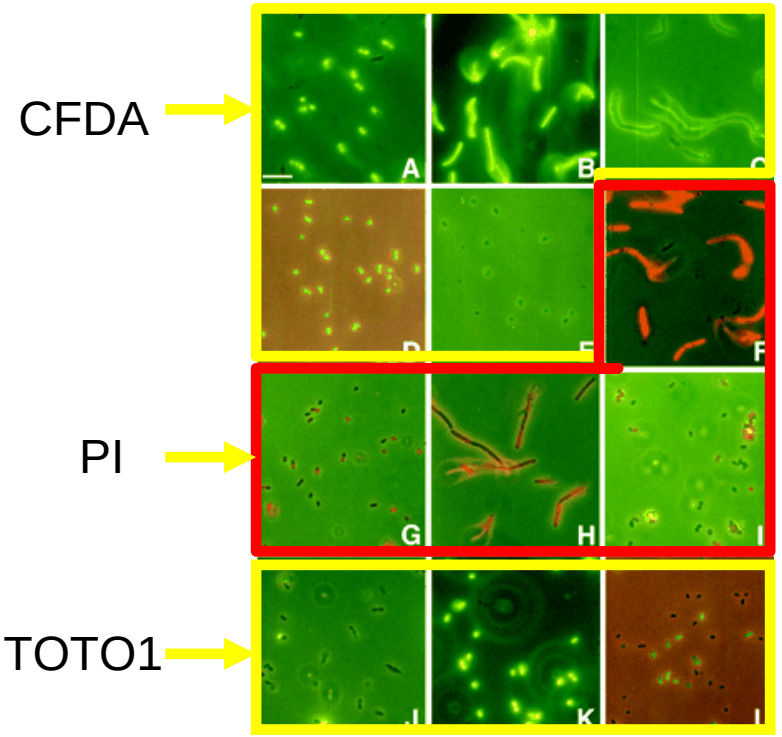


(Bunthof *et al.*, AEM,2001, Vol. 67, No. 5)

Flow Cytometric Assessment
of Viability of Lactic Acid Bacteria



Lactococcus lactis and *Leuconostoc mesenteroides* cell suspensions after exposure to deconjugated bile salts (DBS)

Dehydrogenase activity (respiration)

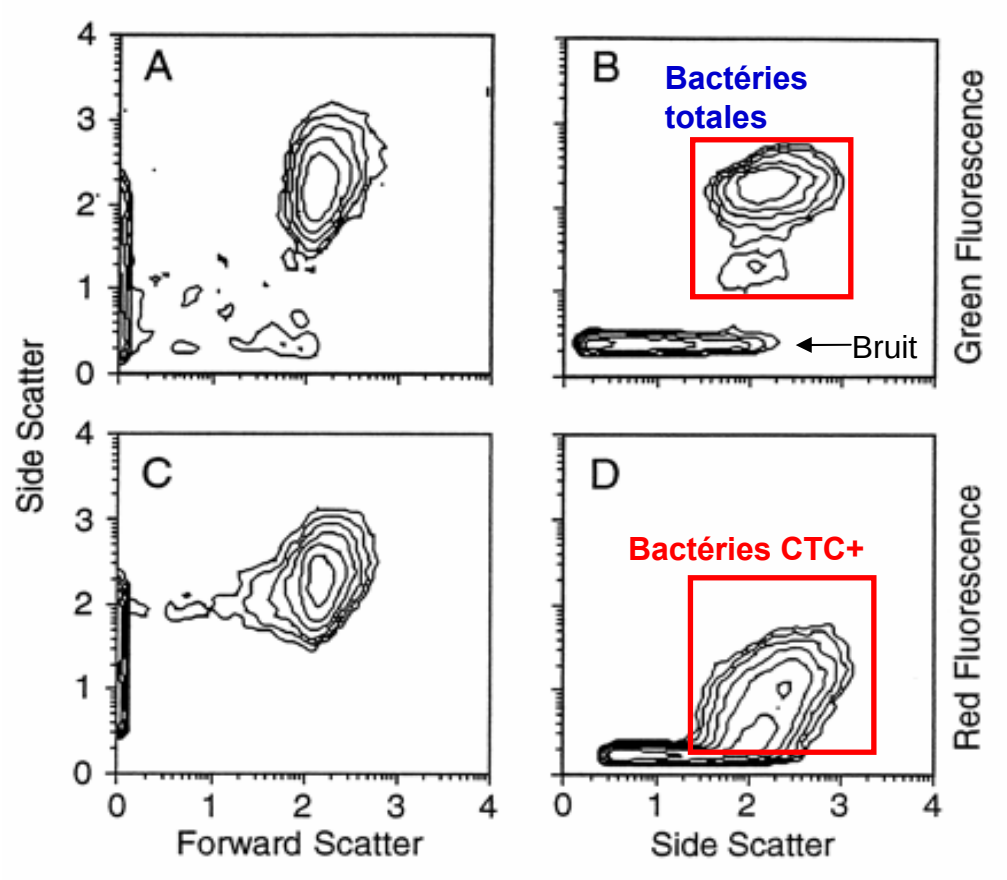
Principle of the CTC:

- 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) is reduced by dehydrogenases (enzymes of the respiratory chain) ➔ red fluorescent precipitate (CTC-formazan)

- Indicates the functionning of the respiratory chain

--- Samples incubated with CTC can be fixed and stored before analysis ---

Analysis by flow cytometry



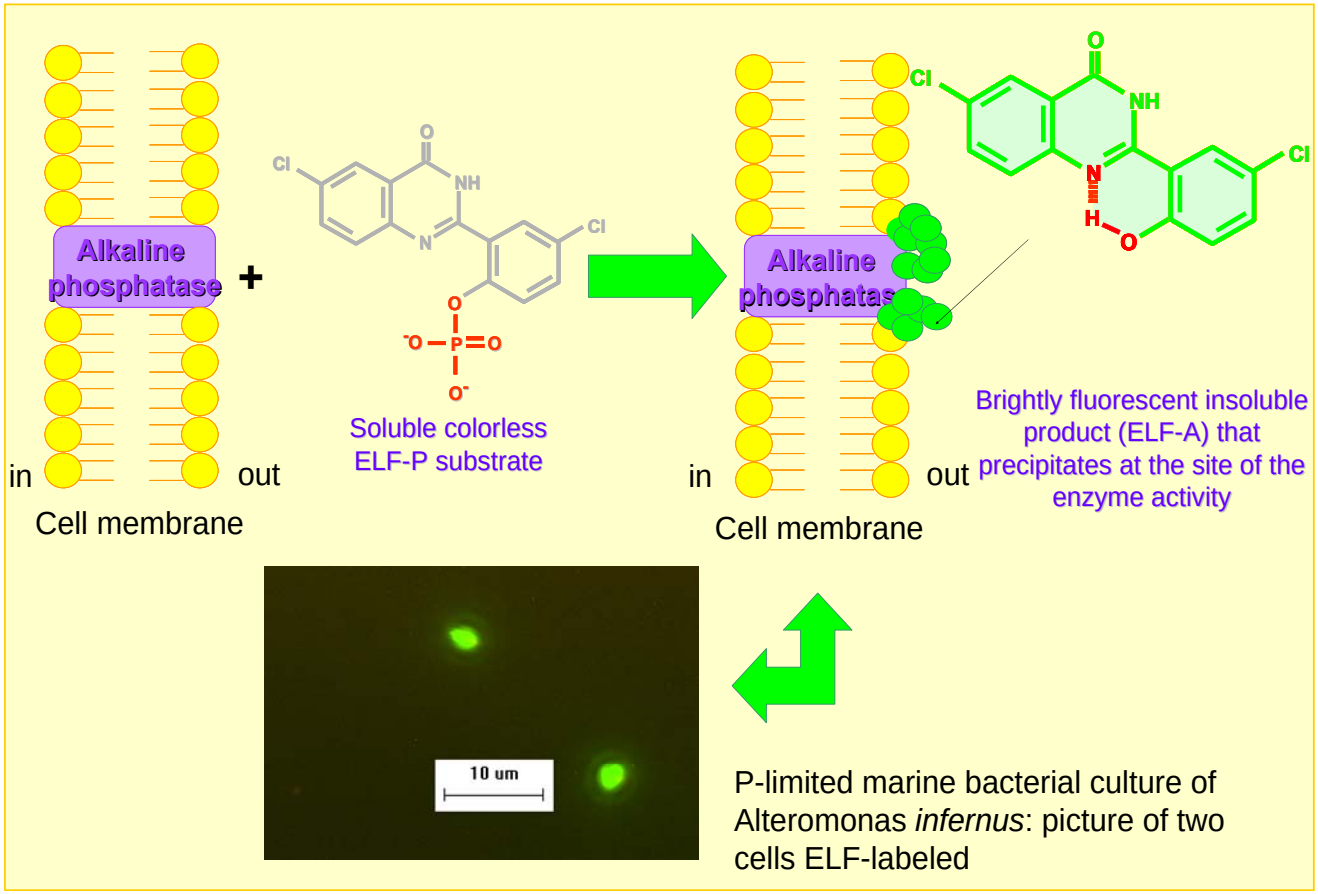
Sieracki *et al.*, AEM, 1999, Vol. 65, No. 6

Cell activity assessed by flow cytometry

Phosphatase activity of heterotrophic bacteria measured at the single cell level

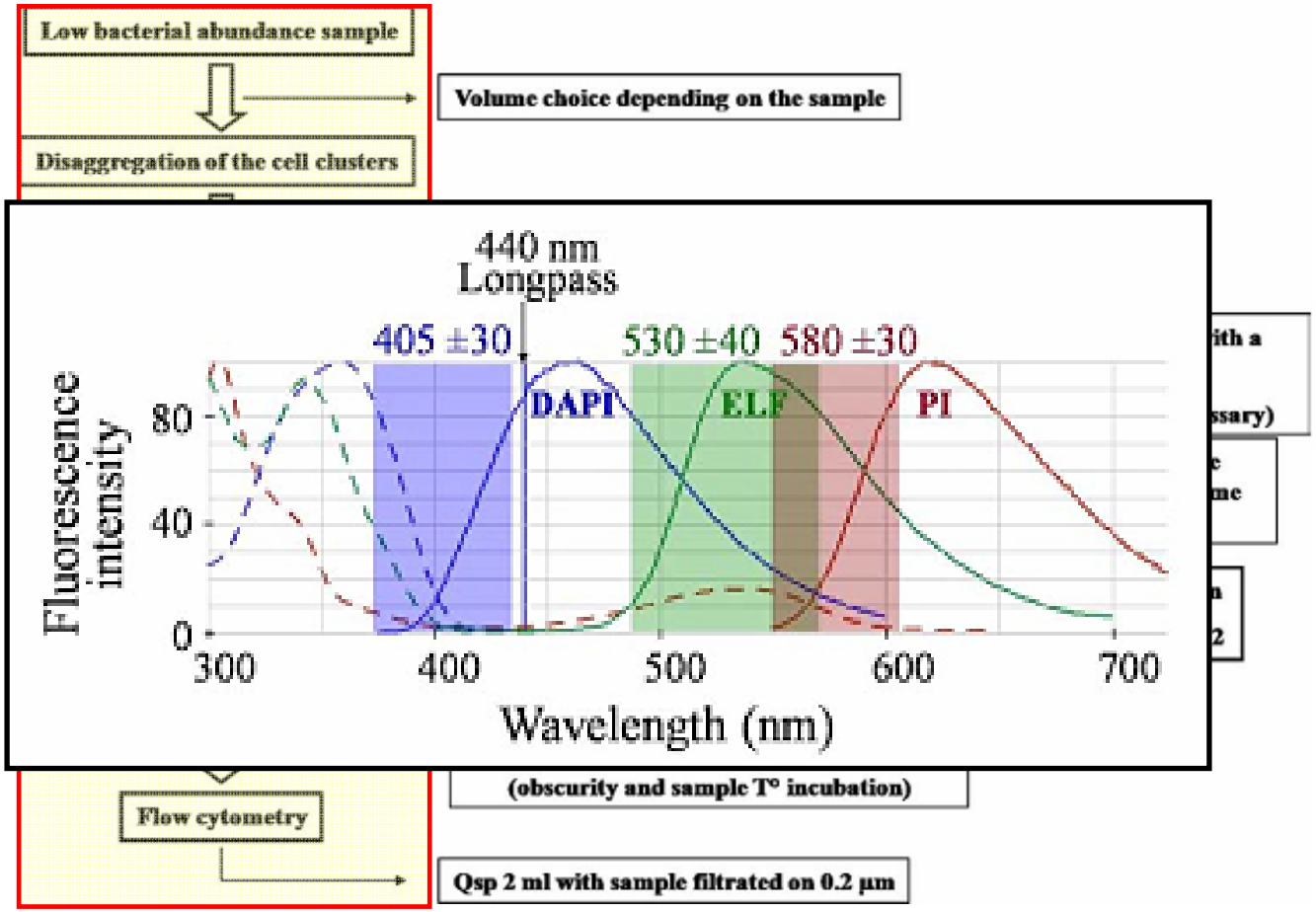
Principle of the method

Based on the ELF 97 fluorochrome (Molecular Probes, USA)



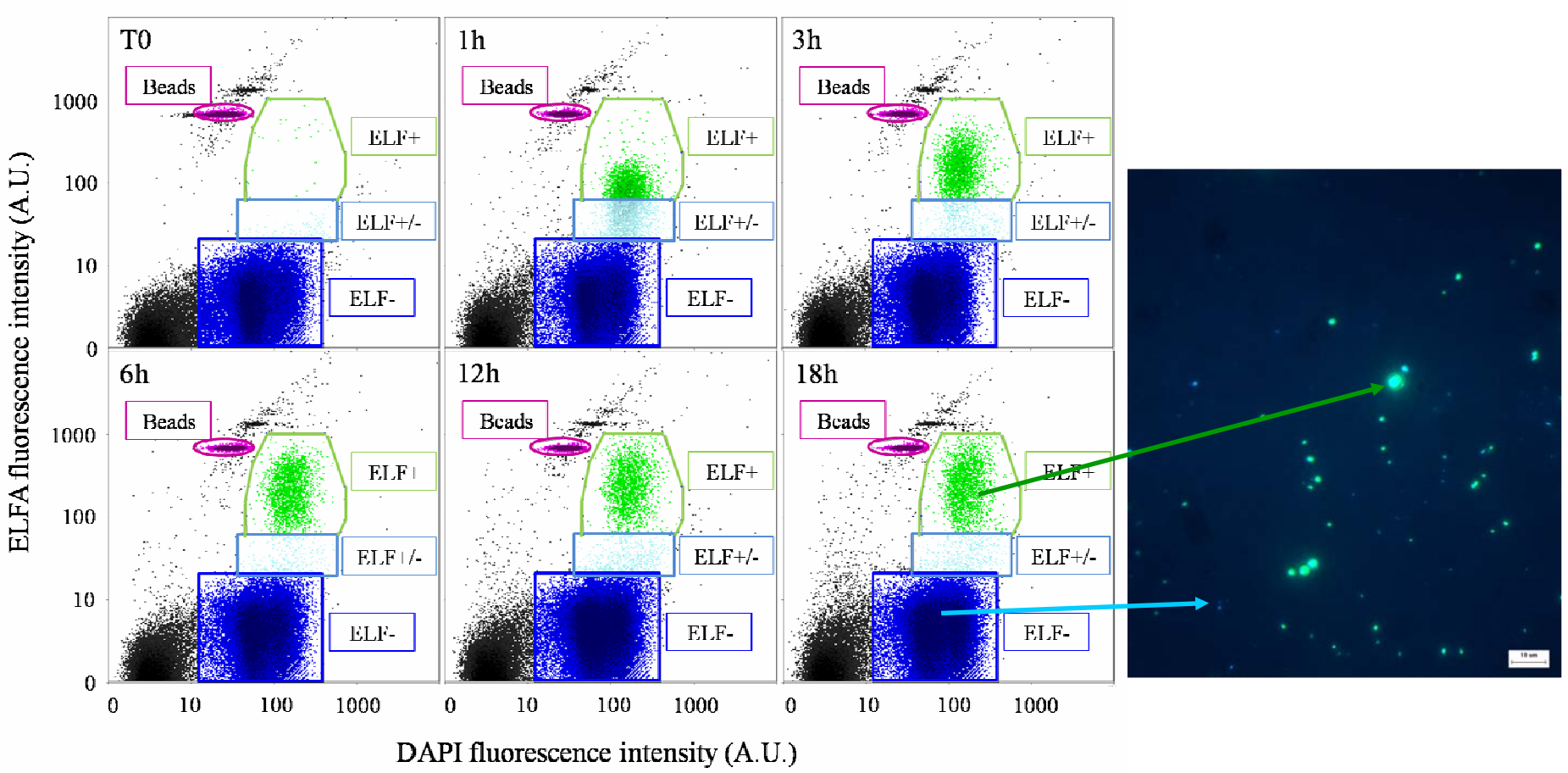
P-limited marine bacterial culture of Alteromonas *infernus*: picture of two cells ELF-labeled

A protocol designed for natural fresh and marine samples



- Duhamel S., Grégori G., Mauriac R., Van wambeke F., Nedoma J. (2008) . J Microbiol Methods 75, 269–278.

Analysis by Flow Cytometry



- Duhamel S., Grégori G., Van Wambeke F., and Nedoma J. (2009). Cytometry 75A Issue 2 : 163 - 168.
- Duhamel S., Grégori G., Van-Wambeke F., Nedoma J. (2009). Current Protocols in Cytometry : Unit 11.18

Direct analysis of multiple genes in individual bacteria

Sequencing multiple DNA loci in individual bacterial cells rather than environmental DNA extracts ➔ high-speed droplet-based cell sorting by flow cytometry

Table 1. Phylogeny of bacterial SSU rRNA genes obtained from single amplified genomes

SAG ID	Lysis protocol	Genus*	Closest isolate†	Closest sequence‡	T-RFL (HhaI), bp	T-RFL (HaeIII), bp
Flavobacteria/Flavobacteriaceae						
MS021–5C	A	*Kordia*, 26	Flavobacterium sp. 3034 AM110988, 91	Clone NorSea37 AM279169, 96	90	283
MS024–2A	B	*Kordia*, 36	Flavobacterium sp. 3034 AM110988, 91	Clone NorSea43 AM279191, 99	94	No cut
MS024–3C	B	*Cellulophaga*, 80	*Cellulophaga* sp. CC12 DQ356487, 93	Clone 1D10 AY274838, 99	96	32
MS024–1F	B	*Tenacibaculum*, 98	Sponge bacterium Zo9 AY948376, 97	Clone WLB13–197 DQ015841, 96	90	281
MS056–2A	C	*Ulvibacter*, 99	*Ulvibacter litoralis* AY243096, 95	Clone PB1.23 DQ071072, 99	94	284
Sphingobacteria/Saprospiraceae						
MS190–1F	B	*Heliscomenobacter*, 55	Saprospiraceae bacterium MS-Wolf2-H AJ786323, 88	Clone SanDiego3-A7 DQ671753, 100	92	407
Alphaproteobacteria/Rhodobacteraceae						
MS056–3A	C	*Sulfitobacter*, 98	*Roseobacter* sp. AY167254, 99	Clone F3C24 AY794157, 100	55	32
MS024–1C	B	*Jannaschia*, 60	*Ophiopholis aculeata* symbiont U63548, 99	Clone EB080-L11F12 AY627365, 100	55	32
MS190–2A	B	*Jannaschia*, 55	*O. aculeata* symbiont U63548, 99	Clone EB080-L11F12 AY627365, 100	55	32
MS190–2F	B	*Loktanella*, 41	*Octadecabacter orientus* KOPRI 13313 DQ167247, 97	Rhodobacteraceae bact. 183 AJ810844.1, 99	55	32
Gammaproteobacteria/Oceanospirillaceae						
MS024–3A	B	*Balneatrix*, 24	Marine gammaproteobacterium HTCC2120 AY386340, 90	Clone Ant4D3 DQ295237, 99	575	413
Gammaproteobacteria/Comamonadaceae						
MS024–2C	B	*Delftia*, 100	*Delftia acidovorans* AM180725, 99	*D. acidovorans* AM180725, 99	203	197

Stepanauskas and Sieracki www.pnas.orgcgidoi10.1073pnas.0700496104

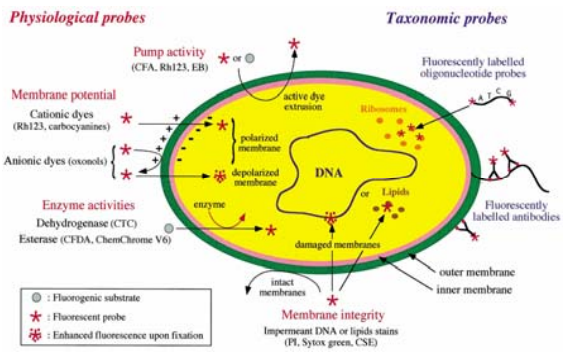
-Microsamples contain the target cell and only 3–10 pl of sample around it

-This reduces the codeposition of extracellular DNA, which in marine waters occurs at concentrations similar to cell-bound DNA.

Toward the single cell analysis

Flow cytometry (& microscopy)

Cell sorting



- **Accumulation of information at the single cell level**
- **Better understanding of the whole community (sociology)**

Discrimination → **Qualification** → **Quantification**

Single cell analysis

Global Scale (ocean)

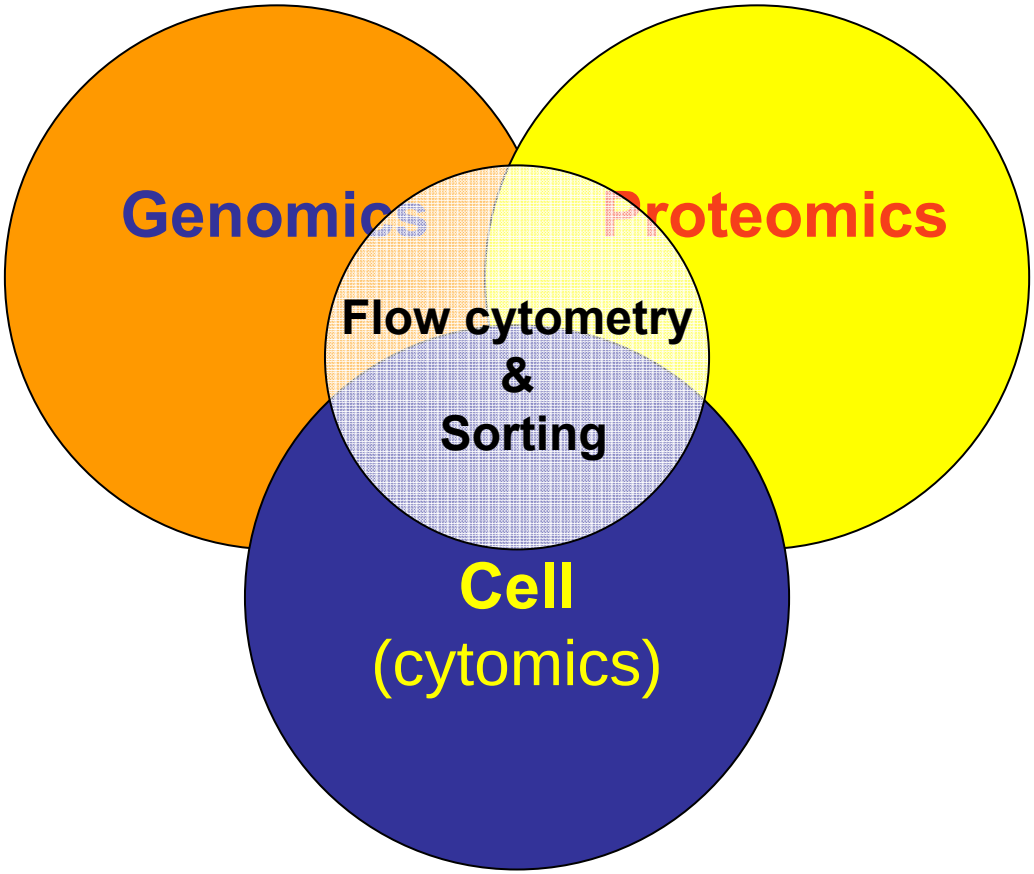
Technical limitations of Flow cytometry

- **Single particles**
  - → Problem of attached cells (i.e., Diatoms)
  - → Size limitation (diameter of the needle)
- **Biomedical origins**
  - → Few plug & play applications
  - → Qualitative versus quantitative analysis
  - → Cell concentrations *in situ* too low (cells>20µm)
  - → Determination of the volume analyzed
  - → Rare events (FCM not suitable)
- **Sensitivity**
  - → Dim fluorescence of surface water algae
  - → Molecular probe fluorescence
- **FCM is a "blind method"**
  - → Controls very important (microscopy)

Special needs for analysis of aquatic samples

- Better discrimination of small particles (sub-micron) such as viruses, or dim particles,
- Accurate volume analyzed (abundances)
- Compact and Robust instruments for work at sea onboard,
- Larger volume analyzed (specially for « big » cells with size > 10 µm),
- Automation of the acquisition
- Automation of the data analysis
- Standardization of the protocols (sampling, fixation, storage)
- Standardization of the data analysis (with standards?)

Conclusion



Structure/function relationship


Thank you for your attention ...
Merci…
谢谢
请指正！